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A New Sterol Sulfate, Sch 572423, from a Marine Sponge, *Topsentia* sp.

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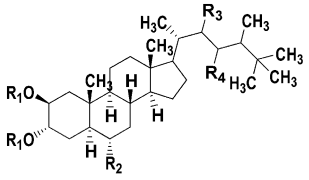
Abstract—Bioassay-guided fractionation of an active fraction from a marine sponge *Topsentia* sp. in our marine fraction library (MFL) led to the isolation and identification of halistanol sulfate (**1**) and a new sterol sulfate Sch 572423 (**2**). Compounds **1** and **2** were identified as $P2Y_{12}$ inhibitors with IC_{50} of 0.48 and 2.2 μM, respectively. The general method of purification for the MFL library and the structure elucidation of compound **2** are described.

Marine organisms are an excellent source of structurally diverse molecules which are potentially useful for drug discovery.[1] Several marine natural products have been discovered as new lead compounds in various therapeutical areas.[2,3] The compounds derived from marine sources currently under clinical studies include bryostatin 1, dolastatin 10, LU-103793, ecteinascidin 743, aplidine (dehydrodidemnin B), discodermolide, TNP-470, and squalamine mainly in the anti-cancer area.[2–4] In our marine natural product research program, we have built a marine fraction library (MFL) for high throughput screening (HTS) assays in different biological targets. The active fractions in various therapeutic areas were followed up using bioassay-guided fractionation to identify the active compounds.

Isolation and identification of active principals from the marine sponge *Topsentia* sp. (Halichondriidae) are described in this paper. In this study, a fraction was identified which bound to the $P2Y_{12}$ receptor. Followed by bioassay-guided fractionation of this active fraction, one new compound Sch 572423 (**2**) was discovered in addition to the principal active compound, halistanol sulfate (**1**).



Halisnol sulfate (**1**) $R_1 = SO_3Na$; $R_2 = OSO_3Na$; $R_3 = R_4 = H$
Sch572423 (**2**) $R_1 = SO_3Na$; $R_2 = H$; $R_3 = R_4 = OH$
Sch599469 (**3**) $R_1 = H$; $R_2 = H$; $R_3 = R_4 = OH$

Purinergic receptors of the P2Y subclass bind naturally occurring nucleotides and signal through G-proteins.[5] $P2Y_1$ and $P2Y_{12}$ receptors are expressed in mammalian platelets. Activation of these receptors by ADP induces platelet aggregation.[6] The molecular identity of the $P2Y_{12}$ receptor, which is the target of the antithrombotic thienopyridine clopidogrel, was recently established.[7,8] Targeted deletion of this receptor gene in mice results in impaired platelet aggregation and prolonged bleeding time.[9] Binding to the $P2Y_{12}$ receptor was evaluated as previously described using the radioligand [^{3}H] 2-methylthio-ADP and 1321N cells stably expressing the receptor.[9]

The sponge was collected using the Johnson-Sea-Link I manned submersible at a depth of 240 feet off Andros Island, Bahamas (latitude 24°38.82′N, longitude 77°41.11′W). The sponge has not been described to the

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species level, however, it is characterized by a plate shape, firm consistency, and cream color. It was approximately 15 cm in diameter and 1.5 cm thick, attached to a rock overhang. The sponge has a dense choanosome and microhispid ectosome. It contains a spicule skeleton of oxeas in several size categories, some of which are bent. The sponge is identified as *Topsentia* sp.[10] (Class: Demospongiae; Order: Halichondrida; Family: Halichondriidae). A reference sample preserved in ethanol has been deposited in the Harbor Branch Oceanographic Museum (catalog number 003:00990, DBMR number 25-XI-92-3-011) and is available for taxonomic evaluation.

In the primary HTS assay, a sample in the MFL from a marine sponge *Topsentia* sp. displaced [^{3}H] 2-Me-S-ADP binding to the $P2Y_{12}$ receptor with 93% displacement at 20 μg/mL. Therefore, the active fraction was subjected to bioassay-guided fractionation. The general procedure for generation of the primary fractions in the marine fraction library was conducted as follows: Crude sample (100–200 g) was ground and extracted with ethanol. The dried organic extract was fractionated on a CG161 column (~60 mL) to generate 6 fractions eluting with aqueous acetonitrile (ACN) gradient solution (10, 25, 50, 75, 100%) and followed by eluting with methanol–EtOAc. All fractions were dried and part of the material was submitted for HTS assay. In this study, fraction 3 (50% ACN elution) was active in the $P2Y_{12}$ binding assay. Fraction 3 (35 mg) was further purified on an HPLC semi-preparative ODS-A column (YMC, 120 Å, S-7, 2×25 cm). The column was eluted with gradient aqueous ACN system (3–50% ACN over 70 min), to yield 100 fractions (13 mL/fraction). Two pure compounds **2** (2.7 mg) and **1** (13 mg) were obtained with retention time ~37 and ~62 min, respectively.

The structure of compound **1** was identified as halistanol sulfate based on detailed NMR and MS analysis.[11] The ion peak of $[M-Na]^-$ of **1** was detected in high-resolution negative FABMS (obsd. *m*/*z* 731.2195; calcd *m*/*z* 731.2182 for $C_{29}H_{49}O_{12}S_3Na_2$). The proton and carbon NMR data analysis further indicated that **1** is identical to halistanol sulfate as reported in the literature.[12]

The structure of compound **2** was determined based on extensive NMR and HRMS analyses and by comparison with compound **1** (Table 1). From the high-resolution negative FABMS, the molecular formula of **2** was established as $C_{29}H_{50}O_{10}S_2Na_2$ (obsd. 645.2758; calcd 645.2744 for $[M-Na]^-$ of $C_{29}H_{50}O_{10}S_2Na$). All spectral data including HRMS, ^{1}H and ^{13}C NMR data strongly suggested that compound **2** was structurally related to **1**, and indicated the presence of two sulfate ester groups in the molecule. The overlapping ^{1}H and ^{13}C signals of three methyl groups indicated the presence of a *t*-butyl group in the molecule. The location of two hydroxyl groups on the side chain was determined by analysis of HMBC and HSQC–TOCSY data. In the HMBC spectrum, *O*-substituted C-22 (δ 75.7) and C-23 (δ 74.1) had three bond H–C couplings with H_3-21 (δ 0.90), and H_3-26 (δ 0.84), respectively. In addition, H–C two-bond correlations observed below from HSQC–TOCSY data allowed us to determine and assign the side-chain structure: H-20/C-17, C-21; H-22/C-23; H-23/C-22; H-24/C-26 (see Fig. 1).

Table 1. NMR spectral data for compounds Sch 572423 (**2**) in CD_3OD

No.	^{1}H (*J*)	^{13}C	HSQC–TOCSY (2 bond)	HMBC (C no.)	NOE correlations from ROESY
1α	1.39, m	39.3 t	C-2		H_3-19
1β	2.07, brd, (14.6)				
2	4.72, br s	76.6 d	C-1		
3	4.69, br s	76.3 d	C-4		
4α	1.60, m	30.6 t	C-5		H_3-19
4β	1.79, td (12.8, 2.2)				
5	1.58, m	40.3 d	C-6		
6	1.26, m	29.3 t	C-5, 7		
7	0.94, m	33.4 t	C-6, 8		
	1.68, m				
8	1.40, m	36.6 d	C-7, 9		H_3-18, H_3-19
9	0.71, m	56.8 d	C-11		
10		36.6 s			
11β					
11α	1.32, m				
	1.52, m	22.2 t	C-9, 12		H_3-18, H_3-19
12α	1.17, m	41.7 t	C-11		H-14
12β	1.99, brd, (12.7)				H_3-18, H_3-21
13		43.9 s			
14	1.08, m	58.1 d	C-8		H-12α, H-17
15β	1.08, m	25.3 t	C-16		
15α	1.60, m				
16β	1.31, m	28.7 t	C-15, 17		H-22
16α	1.90, m				
17	1.49, m	54.1 d	C-20		H-14, H_3-21
18	0.71, s	12.7 q		12, 13, 14, 17	H-11β, H-12β, H-17β, H-20, H_3-21
19	0.99, s	14.5 q		1, 5, 9, 10	H-1β, H-4β, H-8, H-11β
20	1.86, m	37.4 d	C-17, 21		H_3-18, H-23
21	0.90, d, (6.8)	12.4 q		17, 20, 22	H-12β, H-17, H_3-18, H-23
22	3.43, d, (9.7)	75.7 d	C-23	21, 23	H_2-16, H-24, H_3-26
23	3.79, d, (9.7)	74.1 d	C-22	22, 25, 26	H_3-21
24	1.65, m	43.3 d	C-26		H-22, H_3-27, H_3-28, H_3-29
25		34.1 s			
26	0.84, d, (7.1)	8.2, q	C-24	23, 24, 25	H-22, H_3-27, H_3-28, H_3-29
27	0.93, s	29.0 q		24, 25, 28, 29	H-24, H_3-26
28	0.93, s	29.0 q		24, 25, 27, 29	H-24, H_3-26
29	0.93, s	29.0 q		24, 25, 27, 28	H-24, H_3-26

δ in ppm; *J* in Hz.

The sterol skeleton was confirmed using a similar method with analyses of HMBC and HSQC-TOCSY data. The connection of the consecutive protonated carbons in sterol skeleton was completed through two-bond H–C correlations by analysis of HSQC-TOCSY data. The connectivity of quaternary carbons C-10 and C-13 to their neighboring carbons were determined by HMBC through H_3-18 and H_3-19, respectively, as shown in Figure 1. Therefore, the full skeleton of sterol **2** was determined. The two sulfate ester groups were

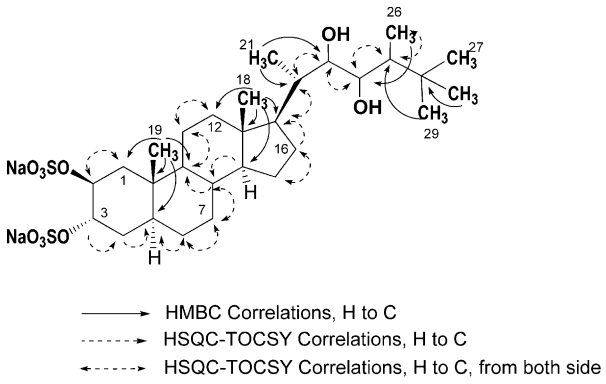


Figure 1.

Table 2. NMR spectral data for compounds Sch 599469 (**3**)

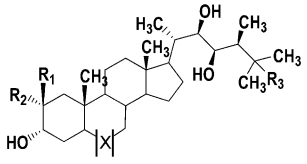
No.	^{1}H (δ)	^{13}C (δ)	No.	^{1}H (δ)	^{13}C (δ)
1	1.51, m 1.73, m	40.5	16	1.31, m 1.88, m	27.7
2	3.91, br s	71.8	17	1.42, m	52.7
3	3.87, br s	70.6	18	0.70, s	12.1
4	1.33, 1.90	31.7	19	0.99, s	14.6
5	1.56, m	38.9	20	1.89, m	36.0
6	1.26, m	29.7	21	0.94, $J=7.4$	11.6
7	0.93, m 1.67, m	31.9	22	3.45, $J=9.2$	73.6
8	1.38, m	35.0	23	3.79, $J=9.2$	70.9
9	0.73, m	55.1	24	1.71, m	42.3
10		35.7	25		33.1
11	1.28, m 1.52, m	20.9	26	0.85, $J=7.5$	7.5
12	1.17, m 1.97, m	40.1	27	0.95, s	28.2
13		42.7	28	0.95, s	28.2
14	1.08, m	56.4	29	0.95, s	28.2
15	1.09, m 1.61, m	24.0			

Recorded in $CDCl_3$; δ in ppm; J in Hz.

assigned to C-2 and C-3 based on their carbon chemical shifts, comparing to those of halistanol sulfate derivatives and the hydrolysis product **3** (Table 2, see below for detail).[12–17]

The stereochemistry of 2- and 3-position was determined as *trans* configuration by the coupling pattern of H-2 (br s) and H-3 (br s). The ^{1}H and ^{13}C chemical shifts and NOE data confirmed the *trans* configuration of all the ring junctions, which were consistent with those of halistanol sulfate (**1**).[12,13] The stereochemistry of side chain of **2** was proposed as 20*S*, 22*R*, 23*R*, and 24*S* based on the ^{1}H–^{1}H J couplings and NOE data with comparison to those of brassinolide (**4**), which stereochemistry was determined by X-ray crystallography.[18] The large J coupling constants of H-22 and H-23 (9.2 Hz) indicated that these two protons had *anti* conformation. The small J couplings ($J\leq1$ Hz) between H-20 and H-22 and between H-23 and H-24 suggested that the dihedral angles were near 90° for both proton pairs. These data were identical to those observed in the side chain of brassinolide ($J=8.4$ Hz for H-22 and H-23; $J\leq1$ Hz for H-20 and H-22 and for H-23 and H-24, respectively).[16,17] The proposed stereochemistry was further supported by the observation of the following NOE correlations shown in the ROESY spectrum: H_3-18 to H-20; H_3-21 to H-23, H-12β, H-17, and H_3-18; H-22 to H-24, H_3-26, H-16α, and H-16β; H-24 to H_3-27, H_3-28, and H_3-29.[14,16,17]

Since compound **2** was insoluble in $CDCl_3$ and most of the NMR data of brassinolide analogues were obtained in $CDCl_3$, hydrolysis product of **2**, Sch 599469 (**3**),[19] was prepared in order to compare NMR data to those of brassinolide analogues in the same solvent. ^{1}H and ^{13}C NMR assignments of compound **3** were accomplished based on extensive spectroscopic data analyses including HMBC, HSQC, and HSQC-TOCSY. The coupling constants, $J=9.2$ Hz for H-22/H-23, $J\leq1$ Hz for H-20/H-22, and $J\leq1$ Hz for H-23/H-24 in compound **3** were virtually identical to those of the synthetic analogue of **4**, 25-methylbrassinolide (**5**), which has the same side chain as those of compounds **2** and **3** ($J=9.0$ Hz for H-22/H-23; $J\leq1$ Hz for H-20/H-22; $J\leq1$ Hz for H-23/H-24, as reported in the leterature for **5**).[20,21] The NOE data obtained from a ROESY experiment for **3** further confirmed the above stereochemistry assignments (NOE: H_3-21 to H-23, H-12β, H-17, and H_3-18; H-22 to H-24, H_3-26, H-16α, and H-16β; H-24 to H_3-27, H_3-28, and H_3-29).



Sch599469 (**3**) R_1 = OH; R_2 = H; R_3 = CH_3; X = CH_2
Brassinolide (**4**) R_1 = H; R_2 = OH; R_3 = H; X = COO
25-Methylbrassinolide (**5**) R_1 = H; R_2 = OH; R_3 = CH_3; X = COO

Compounds **1** and **2** exhibited fairly potent inhibitory activity in the $P2Y_{12}$ assay. The IC_{50} values of **1** and **2** binding to the $P2Y_{12}$ receptor were 0.48 and 2.2 μM, respectively.

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19. Compound **2** (1.2 mg) in 100 μL 1 N HCl, was refluxed for 30 min. The mixture was cooled to rt, and then extracted with EtOAc. EtOAc extract was dried by Na_2SO_4, and after filtration solvent was removed by evaporation. Sch 599469 (**3**, 1 mg) was obtained.
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